

Patterns of BAP1 protein expression provide insights into prognostic significance and the biology of uveal melanoma

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Abstract

Uveal melanoma (UM) is a rare aggressive intraocular tumour with a propensity for liver metastases, occurring in ~50% of patients. The tumour suppressor *BAP1* is considered to be key in UM progression. Herein, we present the largest study to date investigating cellular expression patterns of BAP1 protein in 165 UMs, correlating these patterns to prognosis. Full clinical, histological, genetic, and follow-up data were available for all patients. *BAP1* gene sequencing was performed on a subset of 26 cases. An independent cohort of 14 UMs was examined for comparison. Loss of nuclear BAP1 (nBAP1) protein expression was observed in 54% (88/165) UMs. nBAP1 expression proved to be a significant independent prognostic parameter: it identified two subgroups within monosomy 3 (M3) UM, which are known to have a high risk of metastasis. Strikingly, nBAP1-positive M3 UMs were associated with prolonged survival compared to nBAP1-negative M3 UMs (Log rank, $p = 0.014$). nBAP1 protein loss did not correlate with a *BAP1* mutation in 23% (6/26) of the UMs analysed. Cytoplasmic BAP1 protein (cBAP1) expression was also observed in UM: although appearing 'predominantly diffuse' in most nBAP1-negative UM, a distinct 'focal perinuclear' expression pattern – localized immediately adjacent to the cis Golgi – was seen in 31% (18/59). These tumours tended to carry loss-of-function *BAP1* mutations. Our study demonstrates loss of nBAP1 expression to be the strongest prognostic marker in UM, confirming its importance in UM progression. Our data suggest that non-genetic mechanisms account for nBAP1 loss in a small number of UMs. In addition, we describe a subset of nBAP1-negative UM, in which BAP1 is sequestered in perinuclear bodies, most likely within Golgi, warranting further mechanistic investigation.

Keywords: BAP1; uveal melanoma; prognosis; mutation; immunohistochemistry; cytoplasm; Golgi

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Introduction

The incidence of uveal melanoma (UM) varies between 3 and 8 per million per year in Caucasians, making it the most common primary intraocular

malignancy in adults [1,2]. Whilst primary tumours are successfully treated using either surgery and/or radiotherapy [3], up to 50% of patients die from metastatic disease, typically occurring in the liver, for which there is currently no effective systemic therapy

[4,5]. Stratifying UM patients as at either high or low risk of metastatic spread is critical to ensure patients are managed accordingly: low metastatic risk UM patients can be reassured that they are very unlikely to develop metastases, whilst high metastatic risk UM patients can undergo more intensive liver screening, with the potential of undergoing liver resection surgery at the earliest onset of relapse, and/or enrolment in clinical trials [6]. Prognostication involves the integration of clinical, histological, and genetic features of the tumour, in order to obtain a refined predicted risk [6,7].

UM is characterized by distinct gross chromosomal abnormalities that are associated with patient outcome, the most common being monosomy 3 (M3), which corresponds with a significantly worse prognosis [8–12]. M3 is frequently accompanied by polysomy 8q, and increasing copies of 8q significantly correlate with reduced survival, in a dose-dependent fashion [8,10,13,14]. Further abnormalities described in UM include loss of 1p, which is associated with poor prognosis in the context of M3 [15], and gains on 6p, which, in the absence of additional chromosomal abnormalities, correspond with a good prognosis [16,17]. More recently, mutations occurring in UM have been identified in several genes, including *GNAQ*, *GNA11*, *BAP1*, *SF3B1*, *EIF1AX*, *PLCB4*, and *CYSLTR2* [11,18–22]. In particular, inactivating *BAP1* mutations are associated with a poor outcome, being present in ~84% of all UMs that went on to produce metastases [19,23,24]. *BAP1* mutations similarly correspond with a significantly worse prognosis in renal clear cell carcinoma [25] and cholangiocarcinoma [26], whilst in mesothelioma *BAP1* inactivation is associated with a good prognosis [27]. Families with members carrying germline mutations in *BAP1* have also been described, with individuals most often affected by mesothelioma, melanomas including UM, and renal cell carcinoma [28,29].

BRCA1-associated protein 1 (BAP1), encoded by *BAP1* on chromosome 3p21.1, is a nuclear-localized deubiquitylase (DUB) belonging to the ubiquitin carboxy-terminal hydrolase family of DUBs [19,30]. The 729-amino acid BAP1 protein includes an N-terminal catalytic domain, a bipartite C-terminal nuclear localisation signal (NLS), and binding domains for BRCA1 (Breast Cancer 1), BARD1 (BRCA1-associated RING domain protein 1), and HCF1 (Host cell factor 1), among others [30–32]. BAP1 has multiple functions, including involvement in DNA damage responses [33–35], transcriptional activation [35], chromatin remodelling [36], and cell cycle regulation [33,37].

Functionally, *BAP1* nonsense or frameshift mutations lead to truncated BAP1 protein with loss of nuclear localisation and/or reduced protein or mRNA stability [23]. This has led to several recent studies assessing the prevalence of nuclear BAP1 protein expression (nBAP1) in UM, and correlating this with clinical outcome [23,38,39]. Koopmans *et al* [39] and Kalirai *et al* [38] observed loss of nBAP1 protein expression in 43% and 51% of all UM, respectively, which was significantly associated with increased metastatic risk, along with other clinical features linked to poor prognosis. In the latter study, we also noted the presence of differing cytoplasmic staining patterns of BAP1 protein (cBAP1) using immunohistochemistry (IHC). This has also been observed by groups examining BAP1 IHC expression in other cancers [40–42], and in atypical Spitz tumours [43]. Although the significance of cBAP1 remains unclear, shuttling of BAP1 between the cytoplasm and nucleus has been previously described [44]. Indeed, in cell lines derived from mesothelioma patients, mutant *BAP1* was associated with cytoplasmic sequestration of the protein due to loss of the NLS [45].

Herein, we report the largest study to date of BAP1 protein expression in UM and provide a comprehensive analysis of nBAP1 and cBAP1, correlating these findings with clinical, genetic, and histopathological tumour characteristics, as well as with outcome.

Materials and methods

Ethics

This study conformed to the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. Approval for the study was obtained from the Health Research Authority (NRES REC ref 16/SW/0112), and all patients provided informed consent.

Validation studies were carried out on an independent set of samples obtained from The Ohio State University (OSU) and collected according to a separate IRB (OSU2006C0045).

Sample collection

For samples collected in Liverpool, 4 µm sections were cut from formalin-fixed paraffin-embedded (FFPE) material obtained from 145 UM patients who were treated by enucleation or local resection at the Liverpool Ocular Oncology Centre (LOOC), Royal Liverpool University Hospital NHS Trust between January 2013 and December 2015. For a further 20 patients, BAP1 IHC had already been undertaken as

part of their routine prognostication, and nBAP1 expression data were available from the Ocular Oncology Biobank.

Fourteen samples from OSU were provided as 4 µm sections cut from FFPE blocks.

Immunohistochemistry

The IHC for BAP1 was performed as previously described [38] using the Dako PT Link and the Dako EnVision Flex kit on the Autostainer Plus according to the manufacturer's standard instructions (Dako, Agilent Technologies LDA UK Limited, Stockport, UK). In brief, a mouse anti-human BAP1 antibody (0.5 µg ml⁻¹; C-4, Santa Cruz, Insight Biotechnology Ltd, Middlesex, UK) and a rabbit anti-human GM130 antibody (0.236 µg ml⁻¹; EP892Y, Abcam, Cambridgeshire, UK; a marker for the Golgi apparatus) were used. Bound antibody was visualized with either EnVision™ FLEX 3,3'-diaminobenzidine (DAB+) (Dako) or aminoethylcarbazole (AEC; for highly pigmented tumours) (Vector Labs UK, Peterborough, UK). All sections were counterstained with Mayer's haematoxylin (VWR International Ltd, Luttermouth, UK), blued with Scott's tap water (Leica Microsystems Ltd, Milton Keynes, UK) and mounted with either Aquatex™ (VWR) or DPX mountant for AEC or DAB+ sections, respectively. Human pancreas tissue was used as a positive control for both BAP1 and GM130. Mouse IgG1 was used as a negative control for BAP1 in tissue sections.

Scoring

The IHC stained slides were scored by four independent investigators (SEC, HK, NF, ST) using a light microscope (Nikon Eclipse E600). A binary score was used for nBAP1 expression; with cases being defined as positive when staining was present in tumour cell nuclei, and negative in its absence. cBAP1 was also defined as 'positive' or 'negative', with the subcellular localisation additionally being recorded as either 'predominantly diffuse', or 'predominantly focal perinuclear'.

DNA extraction and quantification

All methods for DNA extraction from FFPE and frozen UM samples and their quantification have been previously described elsewhere [46,47].

Chromosomal copy number analysis

The Multiplex Ligation Dependent Probe Amplification (MLPA) and microsatellite (MSA) procedures

for the assessment of chromosome 3 copy number alterations were performed as previously described [8,48,49]. MLPA was performed in all cases yielding > 100 ng DNA whilst MSA was undertaken for samples with lower DNA yield. For samples obtained from OSU, chromosome 3 status was assessed by microsatellite-based genotyping [46].

Mutation analysis

We hypothesized that the phenomenon of 'focal perinuclear' cBAP1 may be due to distinct *BAP1* mutations resulting in mis-localisation. To study this, we sequenced all *BAP1* exons in 9 UM cases that were nBAP1 positive as well as 17 nBAP1 negative UM (7 cBAP1 diffuse, 9 cBAP1 focal perinuclear, and 1 cBAP1 negative). These cases were chosen because they demonstrated a clear immunophenotype for nBAP1 and also provided good quality DNA. Targeted sequencing of *BAP1* in these cases was performed by the Rotterdam Ocular Melanoma Study group, using the Ion Torrent Personal Genome Machine or by direct Sanger sequencing at OSU [50]. SNPs were called using Torrent Suite Software V4.4.3 (ThermoFisher Scientific Ltd, Hemel Hempstead, UK) and variants were identified and annotated using SnpEff version 3.2a.

A second cohort of 14 UM samples had previously undergone direct sequencing for *BAP1* alterations at OSU. Results were read by aligning with the reference sequence provided by Genbank accession number NM_004656.2, utilizing the Sequencher software (Version 4.8, Gene Codes Corp, Ann Arbor, MI, USA).

Statistical analysis

Multivariate analysis of risk factors associated with development of metastatic disease was undertaken using the Cox proportional hazards model for all covariates with $p \leq 0.10$ by univariate analysis. Survival time (months) was calculated from the date of first diagnosis until death, or study closure on 28 February 2017. All analyses were carried out using SPSS Statistics v.24 (IBM).

Results

Patient demographics and tumour sample details

Initial analyses relate to 165 UM patients treated with surgical excision at the LOOC between January 2013 and December 2015 (supplementary material, Table S1); 154 enucleation, 9 local resection and 2

endoresections. In 29 cases, the surgical excision analysed was not the primary treatment, but instead was performed when tumour recurrence or local complications had followed first treatment. The median time from first presentation to surgical excision for these cases was 33.5 months (range 1–570).

Of the 165 patients, 96 were males and 69 females, with a median age of 63 years at primary management (range, 20–88 years) and a median follow-up of 31.3 months (range, 5.7–348.5 months). At the time of study closure (28 February 2017), 133/165 (81%) UM patients were alive; 25/165 (15%) patients had died from metastatic disease, and 7/165 (4%) had died from other causes. The median largest basal diameter (LBD) was 14.4 mm (range, 3.3–23.6 mm), with a median tumour height of 8.3 mm (range, 0.1–18.5 mm). Epithelioid cells were present in 104/165 (63%) UM, 61/165 (37%) tumours involved the ciliary body, and 20/165 (12%) had extraocular extension. Further histomorphological characteristics of the tumours are summarized in supplementary material, Table S1.

As determined by either MLPA (153 cases) or MSA (12 cases), 103/165 (62%) UM were classified as M3, 48/165 (29%) UM as disomy 3 (D3), and one UM (1%) was isodisomy 3; in all subsequent statistical analyses, this was classified with the M3 cases (supplementary material, Table S2). Thirteen UM (8%) could not be classified as either M3 or D3 due to poor quality DNA.

Nuclear BAP1 protein localisation was observed in 77/165 (45.5%) UM, and absent in 88/165 (54.5%) (Figure 1 and supplementary material, Tables S1 and S2). There was no significant heterogeneity of nBAP1 protein expression amongst the tumour cells, such that the cases were either clearly nBAP1 positive or negative. Glial cells within the optic nerve as well as within the plexiform cells of the neurological retina were used as intrinsic positive controls for nBAP1 expression (Figure 1A). Pancreas sections served as external controls (Figure 1B). Non-neoplastic uveal melanocytes were positive for nBAP1 expression (Figure 1C). No positive staining was observed in sections incubated with mouse IgG1 instead of the BAP1 antibody.

As expected, the majority of M3 (84/104, 81%) were nBAP1 negative; however, 20/104 (19%) demonstrated nBAP1 positivity (Figure 2A). Of the 48 D3 UM, the majority (46/48, 96%) were nBAP1 positive. Two D3 UM were nBAP1 negative and were subsequently re-tested by MSA to rule out isodisomy 3, which is not detectable by MLPA; both had a D3 classification by MSA. Of interest, 90 UM demonstrated 8q gains with 61/90 (68%) being BAP1

negative (supplementary material, Table S2). Furthermore, of the 25 UM patients who died from metastatic UM, 22 (88%) had nBAP1 negative primary tumours (supplementary material, Table S1). Of the three nBAP1 positive cases in this group, the chromosome 3 status was M3 in one, D3 in one, and unclassifiable in the remaining case.

Survival

Kaplan-Meier survival curves and tables were examined for all primary UMs stratified according to nBAP1 protein expression or chromosome 3 status. Both UMs negative for nBAP1 (Log Rank, $p < 0.001$) and M3 UMs (Log rank, $p = 0.001$) were significantly associated with a reduced survival time (Figure 1D,E). The cases were further subdivided according to their chromosome 3 status (Figure 2A–C). Strikingly, M3/nBAP1 positive UM had a significantly better prognosis than M3/nBAP1 negative tumours (Log rank, $p = 0.014$), which was in fact similar to that of patients with D3/nBAP1 positive UM (Figure 2B).

Univariate and multivariate analysis

Univariate analysis identified the following factors as significantly associated with survival time (supplementary material, Table S3): nBAP1 protein expression, $p \leq 0.001$ (HR 0.173; 95% CI 0.067–0.450); age at primary management, $p = 0.005$ (HR 1.046; 95% CI 1.014–1.080); presence of closed connective loops, $p = 0.003$ (HR 3.596; 95% CI 1.541–8.389); mitotic count per 40 high power field, $p = 0.016$ (HR 1.029; 95% CI 1.005–1.054); epithelioid cell morphology, $p = 0.014$ (HR 3.059; 95% CI 1.257–7.443); tumour height, $p = 0.002$ (HR 1.169; 95% CI 1.058–1.292); LBD, $p = 0.006$ (HR 1.142; 95% CI 1.039–1.255); and chromosome 3 loss, $p = 0.036$ (HR 0.482; 95% CI 0.243–0.955). Multivariate analysis demonstrated only nBAP1 protein expression as an independent factor significantly associated with survival, $p = 0.002$ (HR 0.211; 95% CI 0.079–0.562) in this cohort (Table 1).

Cytoplasmic BAP1 protein expression

We next examined the 145 UM stained by the Liverpool Ocular Oncology Research Group for cytoplasmic expression of BAP1. Eighteen of these were excluded from the analysis due to high levels of pigmentation, which made the cBAP1 localisation difficult to distinguish, so 127 UM were ultimately included in the analysis. cBAP1 staining was not observed in the glial cells of the optic nerve or the plexiform cells of the neurological retina, which

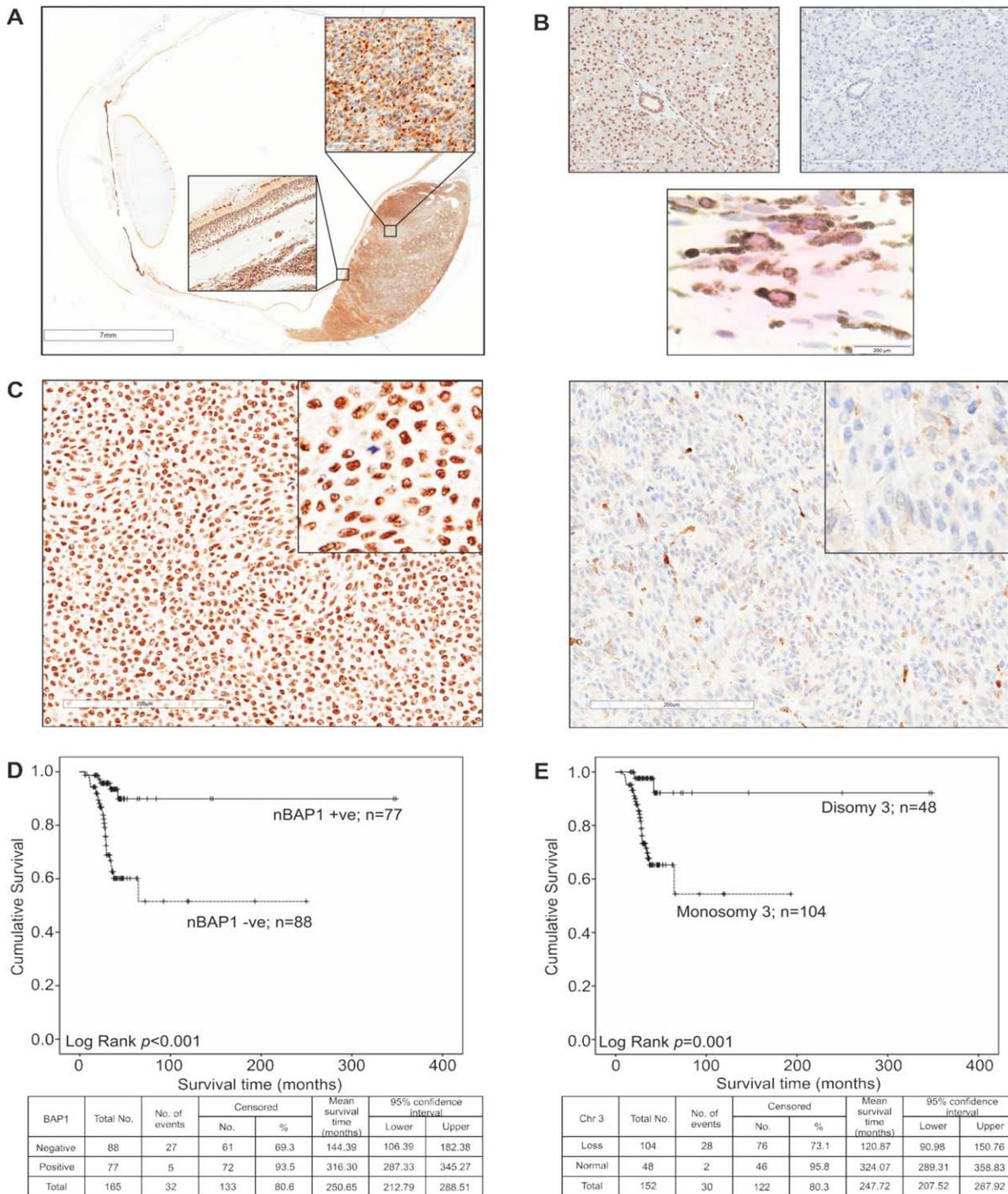


Figure 1. Prognostic value of nuclear BAP1 protein expression. (A–C) Representative images of nuclear BAP1 protein (nBAP1) expression in primary UM (x10 magnification); insets show nBAP1 at x40 magnification. (A) nBAP1 negative tumour with focal perinuclear cBAP1. Intrinsic nuclear positivity is present in the adjacent neural retinal tissue (bottom inset). (B) Positive (left) and negative (right) nBAP1 staining in pancreas control sections. (C) nBAP1 staining in non-neoplastic uveal melanocytes. (D) nBAP1 positive UM (left) and a nBAP1 negative UM (right). (E, F) Kaplan-Meier survival curves and tables for primary UM in the Liverpool cohort stratified according to: (E) nBAP1 protein expression ($n = 165$; Log Rank, $p < 0.001$) (nBAP1 protein expression was scored as positive or negative); or (F) chromosome 3 status ($n = 152$; Log Rank, $p = 0.001$), where only cases with a discernible normal or loss of chromosome 3 were included. Number of events indicates the number of deaths due to metastatic melanoma. Log-rank tests were used to compare survival across groups. BAP1 positivity was detected using a brown chromogen in (A), (B), and (D) and a red chromogen in (C).

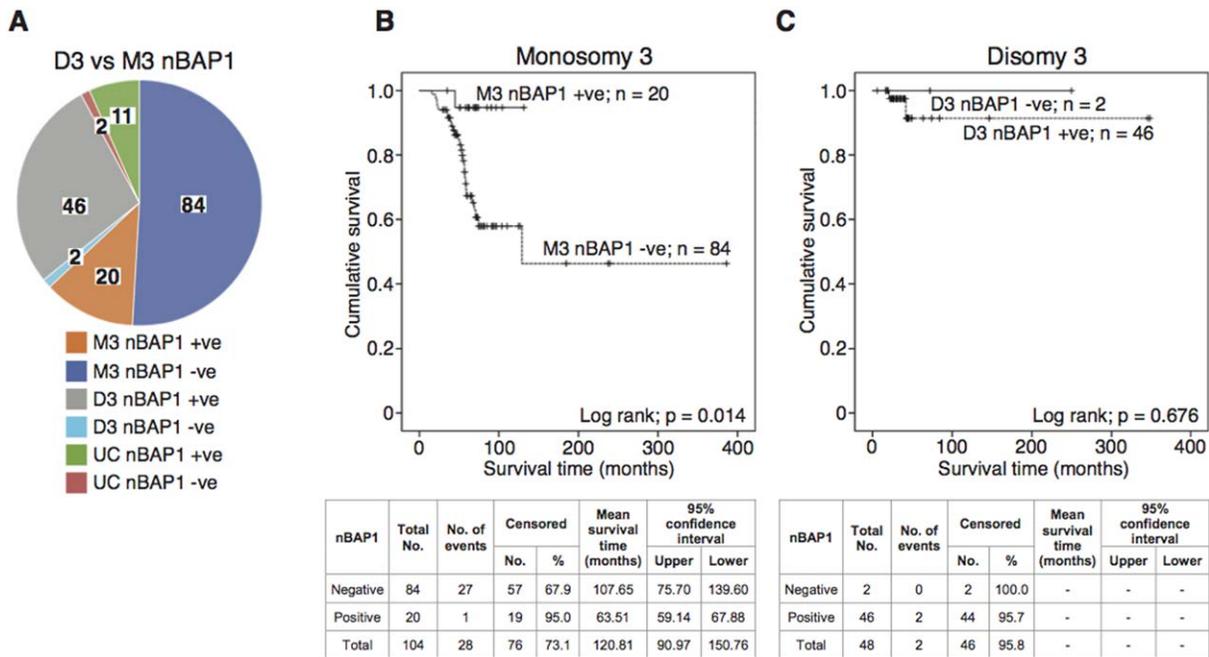


Figure 2. nBAP1 status contributes additional prognostic information in M3-UM patients. (A) Pie chart summarizing the breakdown of nBAP1 staining and chromosome 3 status in the Liverpool cohort of patients, $n = 165$. (B, C) Kaplan-Meier survival curves and tables estimating disease-free survival in UM patients stratified by: (B) nBAP1+ve/-ve status in monosomy 3 (M3) patients, $n = 104$ and (C) nBAP1+ve/-ve status in disomy 3 (D3) patients, $n = 48$. Number of events indicates the number of deaths due to metastatic melanoma. Log-rank tests were used to compare survival across groups.

served as intrinsic positive controls, nor was it detected in mouse IgG1 incubated negative control sections. cBAP1 localisation was recorded as ‘predominantly diffuse’, ‘predominantly focal perinuclear’, or negative. Of the 127 UM examined, 68 (53.5%) were nBAP1 positive, whilst 59 (46.5%) were nBAP1 negative (Figure 3). nBAP1 positive UM demonstrated ‘predominantly diffuse’ cytoplasmic staining in 53/68 (77.9%) cases examined; the remaining 15/68 (22.1%) showed no detectable cBAP1 expression, with none showing the focal perinuclear phenotype.

Of particular interest were the nBAP1 negative UMs that exhibited a distinct ‘focal perinuclear’ cBAP1 localisation in 18/59 (31%) cases (Figure 3A,B). ‘Predominantly diffuse’ cBAP1 was noted in 36/59 (61%) cases and there was no detectable

cBAP1 in 5/59 (8%). Overall, these results suggest that complete loss of BAP1 protein expression is uncommon in UM.

This ‘focal perinuclear’ cBAP1 phenotype did not significantly affect overall survival relative to predominantly diffuse or negative cBAP1 in the nBAP1 negative UM (Log rank, $p = 0.925$; Figure 3C), nor did it correlate significantly with other clinical parameters or gross chromosomal abnormalities.

To take into account possible artefacts created by fixation and processing, we further examined our cBAP1 findings using an external independent cohort of UM ($n = 14$) with available *BAP1* mutational status from the pathology archives of OSU. In keeping with the frequency of nBAP1 loss by IHC in the Liverpool cohort, 7/16 (44%) UM were nBAP1 positive and 9/16 (56%) were nBAP1 negative. All nBAP1 positive UM in this cohort had ‘predominantly diffuse’ cBAP1. nBAP1 negative UM showed ‘focal perinuclear’ cBAP1 localisation in 3/9 (33%) cases with 6/9 (66%) showing ‘predominantly diffuse’ cBAP1 staining, again consistent with our findings above.

Golgi localisation of cytoplasmic BAP1

Our results showing “focal perinuclear” cBAP1 protein accumulation in nBAP1 negative (but not nBAP1

Table 1. Multivariate analysis of risk factors associated with metastasis in uveal melanoma

Variable	Significance	Hazard ratio (HR)	95% CI for HR	
			Lower	Upper
nBAP1	0.006	0.212	0.070	0.645
AgePM	0.071	1.030	0.997	1.064
Chr3	0.974	1.011	0.540	1.892

AgePM, age at primary management; Chr3, Chromosome 3; nBAP1, nuclear BAP1; CI, confidence interval.

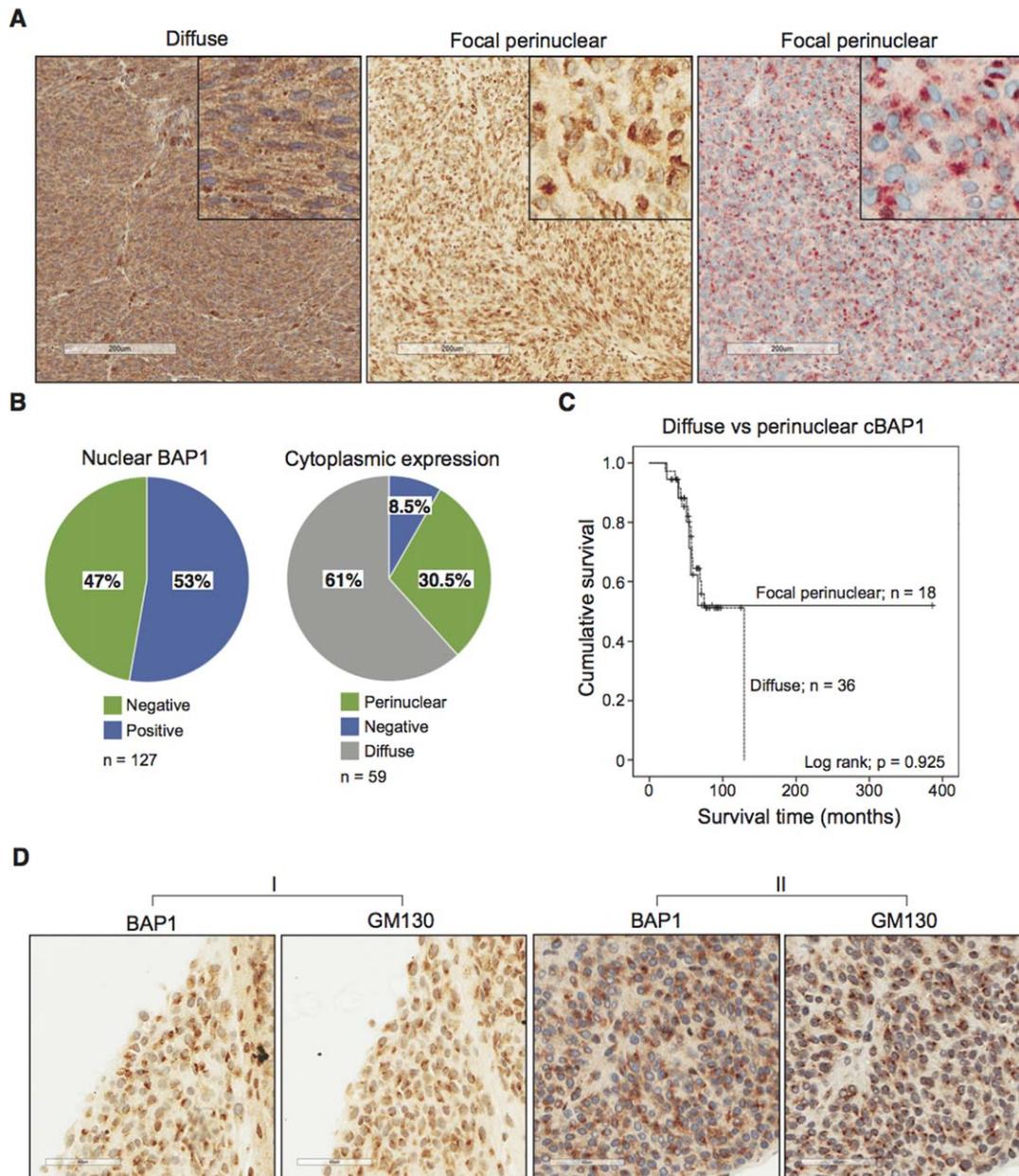


Figure 3. Cytoplasmic BAP1 exhibits differential localisation in UM. (A) Images showing either diffuse (left; DAB) or focal perinuclear (centre; DAB and right; AEC) localisation of cytoplasmic BAP1 (cBAP1) in three nBAP1 negative UM (main image and insets are at $\times 10$ and $\times 40$ magnification, respectively; scale bars 200 μm). (B) Pie charts summarizing the breakdown of nBAP1 expression in the 127 UMs studied (left) and cBAP1 expression in the 59 nBAP1 negative cases (right). (C) Kaplan-Meier survival curve for 54 nBAP1 negative UM patients stratified by focal perinuclear and diffuse cBAP1 staining patterns. The difference in survival was not significant (Log rank; $p = 0.925$). (D) Images showing similar localisation of focal perinuclear cBAP1 with the cis Golgi marker GM130 in 3 μm serial sections of two UM cases (I and II) (scale bars 60 μm).

positive) UM suggests that this reflects aberrant BAP1 processing and/or trafficking. We noted that the perinuclear distribution was similar to the location of the Golgi apparatus: to assess whether the cBAP1 may indeed be accumulating in the Golgi apparatus,

adjacent serial UM sections were stained for BAP1 and the cis Golgi marker, GM130. Interestingly, the GM130 and cBAP1 staining profiles were very similar, indicating that BAP1 is localized near, or possibly within, the cis Golgi in these cells (Figure 3D).

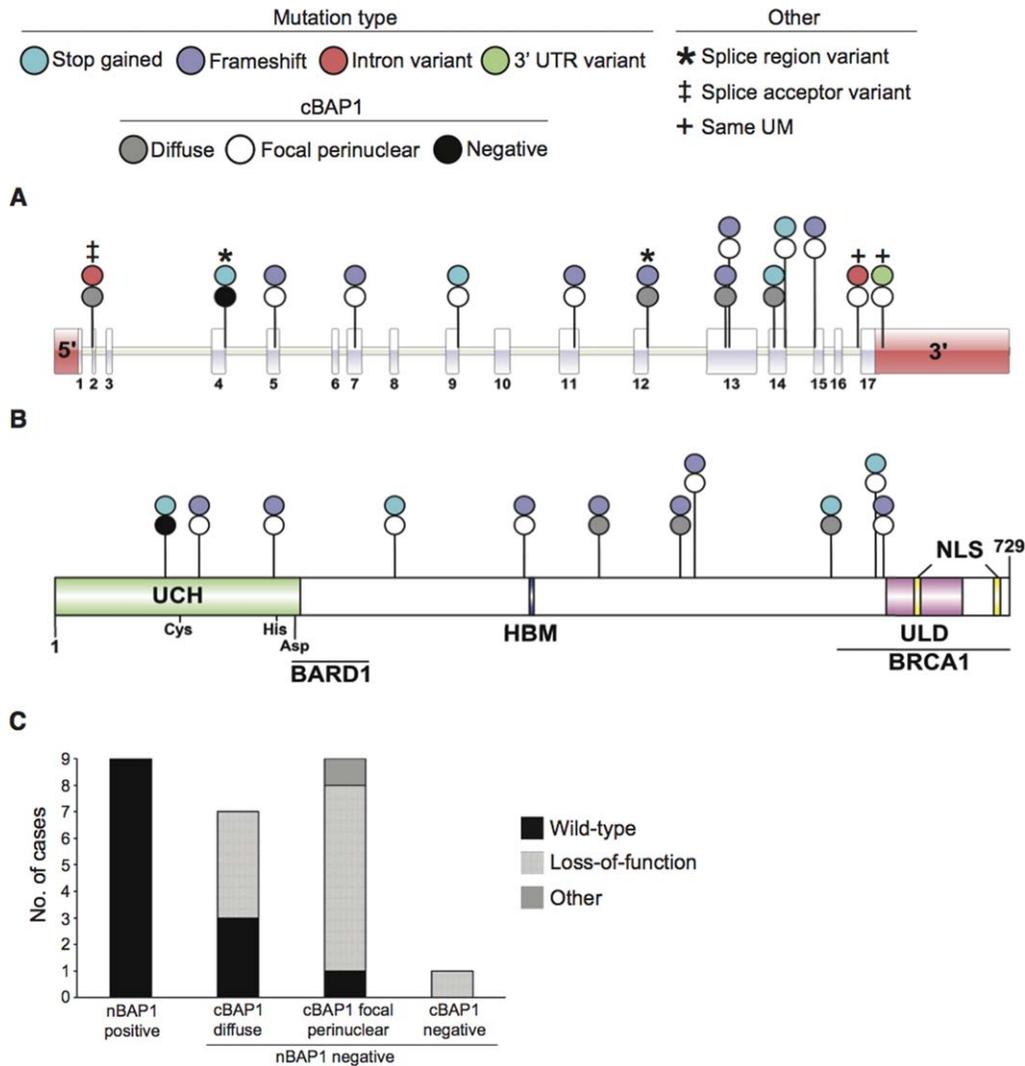


Figure 4. UMs with focal perinuclear cBAP1 tend to harbour loss-of-function *BAP1* mutations. (A) Schematic showing the location of mutations within the *BAP1* gene. 5' and 3' UTRs are depicted in red. One UM had two mutations flanking exon 17 (+); (B) location of the mutations shown in (A) mapped to the BAP1 protein (UCH, ubiquitin carboxy-terminal hydrolase domain; BARD1, BARD1-binding domain; HBM, HCF1-binding domain; BRCA1, BRCA1-binding domain; ULD, UCH-37-like domain; NLS, nuclear localisation signal). The location of cysteine, histidine, and aspartic acid residues of the catalytic triad are shown in the catalytic domain. (C) Histogram showing the distribution of mutation types for the nBAP1 positive, nBAP1 negative (cBAP1 diffuse), nBAP1 negative (cBAP1 focal perinuclear), and nBAP1 negative (cBAP1 negative) UMs.

BAP1 mutation analysis

As expected, all 9 nBAP1 positive UM had wild-type *BAP1*. Of the 17 nBAP1 negative cases examined, 3/7 cBAP1 diffuse UM were wild-type for *BAP1* and 1/7 harboured a splice acceptor mutation prior to exon 2, causing loss-of-function, while 3/7 had truncating, loss-of-function *BAP1* mutations (Figure 4 and supplementary material, Table S4). In contrast, 7/9 cBAP1 'focal perinuclear' UM harboured loss-of-function *BAP1* mutations. In one case, exon 17 was flanked by an intron variant and a 3' UTR variant, the pathogenic significance of which is unclear.

The remaining case was wild-type for *BAP1*. In the independent OSU cohort, 6/9 nBAP1 negative UM harboured a *BAP1* mutation, and 1/9 had a benign variant rs149499021. In the nBAP1 positive UM, 4/5 showed no mutation with only one case having the benign variant rs149499021 [40]. Of the three cases showing 'focal perinuclear' cBAP1, two had INDELS and one had a benign variant (supplementary material, Table S5).

Overall, we identified 6/25 (24%) cases in both cohorts analysed that were nBAP1 negative but did not harbour a *BAP1* mutation.

Discussion

This is the largest study to date examining BAP1 protein expression in UM by IHC. Where feasible, we have also correlated the nuclear and cytoplasmic staining patterns of BAP1 with clinical features, including outcome, as well as with BAP1 sequencing data. In this study, we have demonstrated that nBAP1 protein expression is lost in 54% of UM, and that nBAP1 is a significant independent prognostic indicator, which identifies a subset of M3 UM with nBAP1 expression that have prolonged survival as compared with M3 UM that have lost nBAP1 expression. We provide evidence to suggest that the subcellular localisation of cBAP1 in nBAP1-negative UM is associated with the presence of *BAP1* mutations. That is, UM with 'focal perinuclear' cBAP1 tended to carry loss-of-function *BAP1* mutations compared to UM with diffuse cytoplasmic BAP1.

Nuclear BAP1 findings

BAP1 IHC is currently routinely performed only on surgically resected specimens in our centre. In the current study, this included tumours resected because of size, location, recurrence, secondary complications, and patient preference. Whilst we recognize that there may be a potential bias as compared with an analysis of all UM, the frequency of nBAP1 negativity in this study (54%) is consistent with that reported in the literature (43–58%) [23,38,39]. Similar to these other reports, we demonstrated a correlation between loss of nBAP1 expression and poor prognostic parameters known to be associated with 'high risk' UM – i.e. increasing age at primary management; ciliary body involvement; increasing tumour LBD; large tumour height; epithelioid cell morphology; PAS-positive connective tissue loops; and loss of chromosome 3 [6].

Previous studies have indicated that there is a strong association between the absence of nBAP1 protein expression and mutations in *BAP1* [23,39]. For example, Van de Nes *et al* demonstrated in a cohort of 66 UM that 33/37 (89%) M3 UM had a *BAP1* mutation together with an absence of nBAP1 protein expression [23]. A similar proportion was reported by Koopmans *et al* in their study of 74 UM, with 88% of the M3 UM harbouring a *BAP1* mutation together with loss of nuclear protein expression [39]. In our current study of 26 nBAP1 negative UM, 20 (77%) harboured mutations in *BAP1*, which is similar to the previous reports detailed above.

Of particular interest in our current study was the identification of a subset of M3 UM (20/104; 19%)

that showed nBAP1 positivity, correlating with a significantly increased survival time as compared with the M3/nBAP1-negative UM ($p = 0.014$) (Figure 2B). Whilst the follow-up is relatively short for these 20 individuals, the data support our previous description of a patient with nBAP1 positive liver metastases who was still alive after more than 10 years post enucleation [51]. More recently, BAP1 IHC performed at our centre showed nBAP1 positivity in metastatic UM cells within the liver of a patient who is alive 37 years after diagnosis of the primary UM. This suggests that two distinct subgroups exist within UM with loss of chromosome 3: those M3 UM with a loss of *BAP1* function, and those M3 UM with a retained functional copy of *BAP1*. Indeed, three M3/nBAP1 positive cases were analysed for *BAP1* mutations and found to be wild type. These data were also reflected in the multivariate analysis where nBAP1 was found to be the only independent variable significantly associated with increased risk of developing metastatic disease. Overall, our data strongly suggest that bi-allelic inactivation of *BAP1* is required to influence prognosis; on this basis, we would predict that the loss of other genes on chromosome 3 may have little or no additional effect on prognosis in this group.

In a small number of cases, however, BAP1 loss is clearly not the metastatic driver. For example, 3/26 (12%) UM patients in our study who developed metastatic disease were found to have nBAP1 positive primary tumours. Two of these patients also had unfavourable clinical and histological characteristics, which would have placed them in the 'high risk' category for developing metastatic disease, irrespective of the tumour's nBAP1 status. These other factors included epithelioid cell morphology, high mitotic count, large tumour size, 8q gain, ciliary body involvement, and extraocular growth [3]. The remaining case, however, is unusual in that it was D3 with clinical and histological features of a low metastatic risk tumour. Previous studies have suggested that D3 UM with a mutation in splicing Factor 3B Subunit 1A (*SF3B1*) have an increased risk of developing metastases [52]. Further studies are necessary to determine whether such a mutation is present in this particular case.

Other unusual cases in our patient cohort included three nBAP1 negative UM with no corresponding loss of chromosome 3. Chromosome 3 copy number for all three cases was re-examined by MSA, which is able to detect isodisomy, i.e. loss of heterozygosity due to acquisition of both copies of the chromosome from the same parent. Only one of the three cases showed isodisomy 3 by MSA. The remaining two

cases were tested for *BAP1* mutations, but were found to be wild type, suggesting alternative mechanisms of nBAP1 loss.

Cytoplasmic *BAP1* expression findings

Our study is the first to characterize cBAP1 in UM by IHC, and the first to investigate the link between cBAP1 genotype and phenotype. Our observation of 'focal perinuclear' cBAP1 localisation is consistent with a study by Gammon *et al* [43], who observed 'clumped perinuclear' cBAP1 in 53% of nBAP1 negative sporadic epithelioid Spitz tumours. They further hypothesized that 'focal perinuclear' cBAP1 may be trapped in the Golgi zone, which our experimental findings using the cis Golgi marker, GM130, would support. It should also be mentioned that mouse acites Golgi reactive (MAG) antibodies have been described in some monoclonal antibody preparations, resulting in non-specific cross reactivity with blood group A1 antigens present in patient tissues [53–55]. It is unlikely, given the prevalence of blood group A1 in the population (~35% individuals), however, that MAG antibodies are responsible for the small percentage of cases (11%) with 'focal perinuclear' positivity observed in our study. Moreover, a recent study by Bononi *et al* also described cBAP1 localisation to the endoplasmic reticulum where it regulates Ca^{2+} release to promote apoptosis [56]. In fibroblasts and mesothelial cells derived from individuals with a heterozygous germline *BAP1*^{+/-} mutation, they demonstrated a reduced Ca^{2+} flux that reduced the ability of *BAP1*^{+/-} mutant cells to undergo apoptosis following DNA damage. Taken together, these results suggest that there is indeed a functional role of cBAP1 in particular cancer types requiring further study.

The preponderance of UM with 'focal perinuclear' cBAP1 that harbour truncating mutations is of interest as one might assume that mutations causing premature stop codons would trigger nonsense-mediated decay of the aberrant transcript, so that incorrect protein is not produced. One possibility, however, is that the UM cells bypass the premature stop codon by using alternative splice sites [57] and/or read-through translation [58] to generate misfolded proteins that are not able to exit the Golgi following processing. Consistent with this, Jensen *et al*, who first described BAP1, identified a number of putative N-linked BAP1 glycosylation sites, suggesting that BAP1 may traverse the Golgi during maturation [30]. Bhattacharya and colleagues [59] suggested an alternative mechanism for the observation of 'focal perinuclear' cBAP1 in the form of cBAP1 aggregates promoted by *BAP1* mutations, which expose hydrophobic

regions of the protein, leading to beta amyloid aggregation. However, their study proposed that specific mis-sense mutations in the catalytic domain were responsible for the observed aggregation, whereas our sequencing results suggest that sporadic truncating mutations throughout *BAP1* can induce the 'focal perinuclear' phenotype. Further studies of the molecular biology of the focal perinuclear phenotype will be warranted.

Our results identifying six nBAP1 negative UM with wild-type *BAP1* is consistent with reports by van de Nes *et al* [23] and Royer-Bertrand *et al* [12], in which five and three cases, respectively, with this phenotype/genotype were described. It is tempting to speculate that a post-translational mechanism, perhaps in the form of UBE2O upregulation, could be responsible for the aberrant cytoplasmic localisation of BAP1 in these cases. The putative E2/E3 hybrid ubiquitin ligase, UBE2O, has been reported to mediate cytoplasmic translocation of BAP1 by multi-monoubiquitylation of the NLS. Auto-deubiquitylation by BAP1 antagonizes the activity of UBE2O, promoting its nuclear retention [44].

Finally, whilst our study found no link between cBAP1 expression and overall survival (Log rank; $p = 0.33$), it is of interest that Zhang *et al* [60] found that high expression of cBAP1 in gliomas was significantly associated with worse overall survival compared to low cBAP1 expression, although there was no mention of any specific localisation of the cBAP1 in this study. A larger cohort of UM with a longer follow-up period is required to ascertain whether a dose-dependent effect of cBAP1 is of clinical relevance.

In summary, we have demonstrated the importance of nBAP1 protein expression as an independent marker of prognosis in patients with UM and that identifies a subpopulation of M3 tumours with favourable survival times compared to their nBAP1-negative counterparts. We also report that exclusion of BAP1 protein from the nucleus is most commonly associated with retention of cBAP1. The molecular mechanisms underpinning 'focal perinuclear' cBAP1 remain unknown, and warrant further study.

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Author contributions statement

NF, ST, SEC, JC, JJS, HK: design of study; NF, ST, YK, CC, MHAR: undertaking the IHC experiments; HH, CC, MHAR: patient care/contribution of patient samples; NF, ST, SEC, HK: evaluation of IHC; ST: undertaking of MLPA/MSA; NF, MHAR: evaluation of sequencing results; AT, NF, ST, HK: evaluation of laboratory data with clinical data and outcomes; All authors: Interpretation of results; All authors: manuscript writing; All authors: critical review of the manuscript.

References

- Virgili G, Gatta G, Ciccolallo L, et al. Incidence of uveal melanoma in Europe. *Ophthalmology* 2007; **114**: 2309–2315.
- Yonekawa Y, Kim IK, Damato B. Epidemiology and management of uveal melanoma. *Hematol Oncol Clin North Am* 2012; **26**: 1169–1184.
- Damato B. Progress in the management of patients with uveal melanoma. The 2012 Ashton Lecture. *Eye (Lond)* 2012; **26**: 1157–1172.
- Carvajal RD, Schwartz GK, Tezel T, et al. Metastatic disease from uveal melanoma: treatment options and future prospects. *Br J Ophthalmol* 2017; **101**: 38–44.
- Woodman SE. Metastatic uveal melanoma: biology and emerging treatments. *Cancer J* 2012; **18**: 148–152.
- Damato B, Eleuteri A, Taktak AFG, et al. Estimating prognosis for survival after treatment of choroidal melanoma. *Prog Retin Eye Res* 2011; **30**: 285–295.
- DeParis SW, Taktak A, Eleuteri A, et al. External validation of the Liverpool uveal melanoma prognosticator online. *Invest Ophthalmol Vis Sci* 2016; **57**: 6116–6122.
- Caines R, Eleuteri A, Kalirai H, et al. Cluster analysis of multiplex ligation-dependent probe amplification data in choroidal melanoma. *Mol Vis* 2015; **21**: 1–11.
- Damato B, Duke C, Coupland SE, et al. Cytogenetics of uveal melanoma: a 7-year clinical experience. *Ophthalmology* 2007; **114**: 1925–1931.
- Prescher G, Bornfeld N, Hirche H, et al. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet* 1996; **347**: 1222–1225.
- Robertson AG, Shih J, Yau C, et al. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. *Cancer Cell* 2017; **32**: 204–220.
- Royer-Bertrand B, Torsello M, Rimoldi D, et al. Comprehensive genetic landscape of uveal melanoma by whole-genome sequencing. *Am J Hum Genet* 2016; **99**: 1190–1198.
- Horsman DE, Sroka H, Rootman J, et al. Monosomy 3 and isochromosome 8q in a uveal melanoma. *Cancer Genet Cytogenet* 1990; **45**: 249–253.
- Sisley K, Cottam DW, Rennie IG, et al. Non-random abnormalities of chromosomes 3, 6, and 8 associated with posterior uveal melanoma. *Genes, Chromosomes Cancer* 1992; **5**: 197–200.
- Kilic E, Naus NC, van Gils W, et al. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci* 2005; **46**: 2253–2257.
- Damato B, Dopierala J, Klaasen A, et al. Multiplex ligation-dependent probe amplification of uveal melanoma: correlation with metastatic death. *Invest Ophthalmol Vis Sci* 2009; **50**: 3048–3055.
- Häusler T, Stang A, Anastassiou G, et al. Loss of heterozygosity of 1p in uveal melanomas with monosomy 3. *Int J Cancer* 2005; **116**: 909–913.
- Dono M, Angelini G, Cecconi M, et al. Mutation frequencies of GNAQ, GNA11, BAP1, SF3B1, EIF1AX and TERT in uveal melanoma: detection of an activating mutation in the TERT gene promoter in a single case of uveal melanoma. *Br J Cancer* 2014; **110**: 1058–1065.
- Harbour JW, Onken MD, Roberson EDO, et al. Frequent mutation of BAP1 in metastasizing uveal melanomas. *Science* 2010; **330**: 1410–1413.
- Martin M, Maßhöfer L, Temming P, et al. Exome sequencing identifies recurrent somatic mutations in EIF1AX and SF3B1 in uveal melanoma with disomy 3. *Nat Genet* 2013; **45**: 933–936.
- Van Raamsdonk CD, Bezroukove V, Green G, et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature* 2009; **457**: 599–602.
- Van Raamsdonk CD, Griewank KG, Crosby MB, et al. Mutations in GNA11 in uveal melanoma. *N Engl J Med* 2010; **363**: 2191–2199.
- van de Nes JAP, Nelles J, Kreis S, et al. Comparing the prognostic value of BAP1 mutation pattern, chromosome 3 status, and BAP1 immunohistochemistry in uveal melanoma. *Am J Surg Pathol* 2016; **40**: 796–805.
- van Essen TH, van Pelt SI, Versluis M, et al. Prognostic parameters in uveal melanoma and their association with BAP1 expression. *Br J Ophthalmol* 2014; **98**: 1738–1743.
- Cancer Genome Atlas Research Network. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* 2013; **499**: 43–49.

26. Al-Shamsi HO, Anand D, Shroff RT, *et al.* BRCA-associated protein 1 mutant cholangiocarcinoma: an aggressive disease subtype. *J Gastrointest Oncol* 2016; **7**: 556–561.
27. Righi L, Duregon E, Vatrano S, *et al.* BRCA1-associated protein 1 (BAP1) immunohistochemical expression as a diagnostic tool in malignant pleural mesothelioma classification: a large retrospective study. *J Thorac Oncol* 2016; **11**: 2006–2017.
28. Rai K, Pilarski R, Boru G, *et al.* Germline BAP1 alterations in familial uveal melanoma. *Genes Chromosomes Cancer* 2017; **56**: 168–174.
29. Rai K, Pilarski R, Cebulla CM, *et al.* Comprehensive review of BAP1 tumor predisposition syndrome with report of two new cases. *Clin Genet* 2016; **89**: 285–294.
30. Jensen DE, Proctor M, Marquis ST, *et al.* BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene* 1998; **16**: 1097–1112.
31. Misaghi S, Ottosen S, Izrael-Tomasevic A, *et al.* Association of C-terminal ubiquitin hydrolase BRCA1-associated protein 1 with cell cycle regulator host cell factor 1. *Mol Cell Biol* 2009; **29**: 2181–2192.
32. Nishikawa H, Wu W, Koike A, *et al.* BRCA1-associated protein 1 interferes with BRCA1/BARD1 RING heterodimer activity. *Cancer Res* 2009; **69**: 111–119.
33. Eletr ZM, Wilkinson KD. An emerging model for BAP1's role in regulating cell cycle progression. *Cell Biochem Biophys* 2011; **60**: 3–11.
34. Ismail IH, Davidson R, Gagne J-P, *et al.* Germline mutations in BAP1 impair its function in DNA double-strand break repair. *Cancer Res* 2014; **74**: 4282–4294.
35. Yu H, Mashtalir N, Daou S, *et al.* The ubiquitin carboxyl hydrolase BAP1 forms a ternary complex with YY1 and HCF-1 and is a critical regulator of gene expression. *Mol Cell Biol* 2010; **30**: 5071–5085.
36. Sahtoe DD, van Dijk WJ, Ekkebus R, *et al.* BAP1/ASXL1 recruitment and activation for H2A deubiquitination. *Nat Commun* 2016; **7**: 10292.
37. Machida YJ, Machida Y, Vashisht AA, *et al.* The deubiquitinating enzyme BAP1 regulates cell growth via interaction with HCF-1. *J Biol Chem* 2009; **284**: 34179–34188.
38. Kalirai H, Dodson A, Faqir S, *et al.* Lack of BAP1 protein expression in uveal melanoma is associated with increased metastatic risk and has utility in routine prognostic testing. *Br J Cancer* 2014; **111**: 1373–1380.
39. Koopmans AE, Verdijk RM, Brouwer RWW, *et al.* Clinical significance of immunohistochemistry for detection of BAP1 mutations in uveal melanoma. *Mod Pathol* 2014; **27**: 1321–1330.
40. Pilarski R, Cebulla CM, Massengill JB, *et al.* Expanding the clinical phenotype of hereditary BAP1 cancer predisposition syndrome, reporting three new cases. *Genes Chromosomes Cancer* 2014; **53**: 177–182.
41. Piris A, Mihm MC Jr, Hoang MP. BAP1 and BRAFV600E expression in benign and malignant melanocytic proliferations. *Hum Pathol* 2015; **46**: 239–245.
42. Testa JR, Cheung M, Pei J, *et al.* Germline BAP1 mutations predispose to malignant mesothelioma. *Nat Genet* 2011; **43**: 1022–1025.
43. Gammon B, Traczyk TN, Gerami P. Clumped perinuclear BAP1 expression is a frequent finding in sporadic epithelioid Spitz tumors. *J Cutan Pathol* 2013; **40**: 538–542.
44. Mashtalir N, Daou S, Barbour H, *et al.* Autodeubiquitination protects the tumor suppressor BAP1 from cytoplasmic sequestration mediated by the atypical ubiquitin ligase UBE2O. *Mol Cell* 2014; **54**: 392–406.
45. Hakiri S, Osada H, Ishiguro F, *et al.* Functional differences between wild-type and mutant-type BRCA1-associated protein 1 tumor suppressor against malignant mesothelioma cells. *Cancer Sci* 2015; **106**: 990–999.
46. Abdel-Rahman MH, Cebulla CM, Verma V, *et al.* Monosomy 3 status of uveal melanoma metastases is associated with rapidly progressive tumors and short survival. *Exp Eye Res* 2012; **100**: 26–31.
47. Lake SL, Kalirai H, Dopierala J, *et al.* Comparison of formalin-fixed and snap-frozen samples analyzed by multiplex ligation-dependent probe amplification for prognostic testing in uveal melanoma. *Invest Ophthalmol Vis Sci* 2012; **53**: 2647–2652.
48. Dopierala J, Damato BE, Lake SL, *et al.* Genetic heterogeneity in uveal melanoma assessed by multiplex ligation-dependent probe amplification. *Invest Ophthalmol Vis Sci* 2010; **51**: 4898–4905.
49. Tschentscher F, Prescher G, Horsman DE, *et al.* Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res* 2001; **61**: 3439–3442.
50. Abdel-Rahman MH, Pilarski R, Cebulla CM, *et al.* Germline BAP1 mutation predisposes to uveal melanoma, lung adenocarcinoma, meningioma, and other cancers. *J Med Genet* 2011; **48**: 856–859.
51. McCarthy C, Kalirai H, Lake SL, *et al.* Insights into genetic alterations of liver metastases from uveal melanoma. *Pigment Cell Melanoma Res* 2016; **29**: 60–67.
52. Yavuziyigitoglu S, Koopmans AE, Verdijk RM, *et al.* Uveal melanomas with SF3B1 mutations: a distinct subclass associated with late-onset metastases. *Ophthalmology* 2016; **123**: 1118–1128.
53. Finstad CL, Yin BW, Gordon CM, *et al.* Some monoclonal antibody reagents (C219 and JSB-1) to P-glycoprotein contain antibodies to blood group A carbohydrate determinants: a problem of quality control for immunohistochemical analysis. *J Histochem Cytochem* 1991; **39**: 1603–1610.
54. Kliman HJ, Feinberg RF, Schwartz LB, *et al.* A mucin-like glycoprotein identified by MAG (mouse ascites Golgi) antibodies. Menstrual cycle-dependent localization in human endometrium. *Am J Pathol* 1995; **146**: 166–181.
55. Ouwendijk WJD, Flowerdew SE, Wick D, *et al.* Immunohistochemical detection of intra-neuronal VZV proteins in snap-frozen human ganglia is confounded by antibodies directed against blood group A1-associated antigens. *J Neurovirol* 2012; **18**: 172–180.
56. Bononi A, Giorgi C, Patergnani S, *et al.* BAP1 regulates IP3R3-mediated Ca²⁺ flux to mitochondria suppressing cell transformation. *Nature* 2017; **546**: 549–553.
57. Dvinge H, Kim E, Abdel-Wahab O, *et al.* RNA splicing factors as oncoproteins and tumour suppressors. *Nat Rev Cancer* 2016; **16**: 413–430.

58. Schueren F, Thoms S, Brosius J. Functional translational read-through: a systems biology perspective. *PLoS Genet* 2016; **12**: e1006196.
59. Bhattacharya S, Hanpude P, Maiti TK. Cancer associated missense mutations in BAP1 catalytic domain induce amyloidogenic aggregation: a new insight in enzymatic inactivation. *Sci Rep* 2016; **5**: 18462.
60. Zhang X-K, Xi S-Y, Sai KE, et al. Cytoplasmic expression of BAP1 as an independent prognostic biomarker for patients with gliomas. *Int J Clin Exp Pathol* 2015; **8**: 5035–5043.

SUPPLEMENTARY MATERIAL ONLINE

Table S1. Association between nBAP1 status and clinical and pathological risk factors in the Liverpool uveal melanoma cohort

Table S2. Association between nBAP1 status and genetic factors in the Liverpool uveal melanoma cohort

Table S3. Univariate analysis of risk factors associated with metastatic disease in uveal melanoma

Table S4. Mutation results from the Liverpool Ocular Oncology Centre cohort

Table S5. Mutation results from the Ohio cohort